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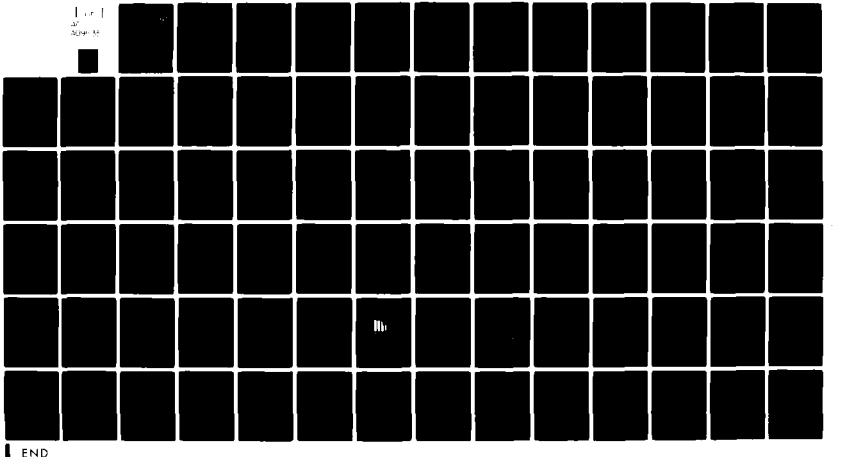

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ROLE OF LYSOSOMAL ENZYME RELEASE IN CIRCULATORY SHOCK AND CRITICAL ILLNESS

Final Report

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Stephen L. Wangensteen, M.D.

June 1978

(For the period May 1973 - September 1976)

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Department of Surgery
University of Virginia School of Medicine
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To determine the association of lysosomal enzyme release and the severity of shock in the human system in states of circulatory shock and conditions of critical illness. We will be studying (1) the release of lysosomal enzymes in several forms of clinical trauma, and (2) the role of administration of agents that may modify the release of lysosomal enzymes.

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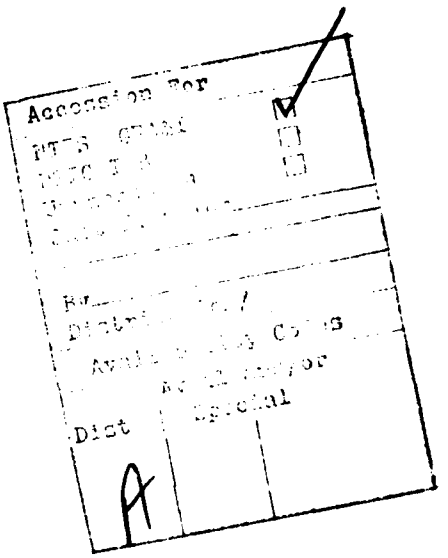
SUMMARY

To determine the association of lysosomal enzyme release and the severity of shock in the human system in states of circulatory shock, studies were carried out on (1) the release of lysosomal enzymes in several forms of clinical trauma and (2) the role of administration of agents that may modify the release of lysosomal enzymes. The appearance of cathepsin D in the circulation of experimental animals subjected to hemorrhagic shock is predictable and reflects the duration and severity of shock. Increases in plasma cathepsin D activity occur early in the course of experimental hemorrhagic shock, suggesting that disruption of normal lysosomal membrane function is an early event in the progression of circulatory shock. The results, however, do not show any adverse effects from the introduction of exogenous cathepsin D into the systemic circulation during hemorrhagic shock and cathepsin D would appear to be inactivated at pH of blood normally found in shock.



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I. INTRODUCTION

The basic cellular changes which occur in response to circulatory shock, and the significance of these changes in terms of pathophysiology, are at present poorly understood. However, one of the effects observed to follow the onset of circulatory shock is the presence of both morphologic and enzymatic alterations in lysosomes, resulting in the release of hydrolytic lysosomal enzymes. Much speculation has been raised concerning the possible role these hydrolytic enzymes might play in the pathophysiology of this complex syndrome and experimental evidence is now available which suggests that these enzymes cause significant damaging effects following their release from lysosomes. Their liberation may therefore constitute a critical event in the shock syndrome, serving as a factor in the lethal outcome of shock.

Cathepsin D, a potent lysosomal acid protease, has been widely implicated as a mediator of injurious effects in circulatory shock. However, adequate direct experimental evidence for deleterious effects of this enzyme is lacking. The present study is an attempt to define adequately the actions of cathepsin D following its introduction into the circulation during hemorrhagic shock.

The Lysosome: Definition and Normal Functions

Lysosomes, first described by de Duve et al in 1955,[1] are a class of subcellular organelles which contain a variety of hydrolytic enzymes. These organelles normally

function as the cytoplasmic vacuolar digestive system and their associated enzymes provide for degradation of substances during the processes of autophagy and heterophagy.[2,3] Under physiologic conditions, lysosomal enzymatic action is controlled by a single limiting lipoprotein membrane. This membrane functionally isolates lysosomal enzymes from their surrounding cytoplasm and equips the organelle with a structure-linked latency which prevents autolysis.[3,4]

The normal intracellular functions of lysosomes are depicted in figure 1. The various lysosomal hydrolases are produced in the rough endoplasmic reticulum[5] and are then transported to the golgi apparatus where they are enclosed by a single membrane.[6] Those lysosomes which have been newly formed in the golgi complex and have not participated in intracellular digestion are termed storage granules or primary lysosomes. During autophagy and heterophagy the primary lysosome fuses with substrate containing organelles to form an enzyme-substrate system which is responsible for intracellular digestion.[7]

Autophagy is the mechanism by which cells rid themselves of damaged organelles and metabolic wastes. In this process cytoplasmic constituents are sequestered into membrane granules, termed autophagic vacuoles or autophagosomes.[2] The autophagic vacuole may fuse with a primary lysosome to form a digestive vacuole or secondary lysosome which contains both substrates and hydrolytic enzymes. Following fusion, extensive hydrolytic enzymatic digestion occurs. Those breakdown

products which are small enough to pass through the lysosomal membrane diffuse into the cytoplasm where they may be utilized for various metabolic and biosynthetic processes.[5]

Secondary lysosomes are capable of successive acts of fusion and digestion; after each digestive process, products are liberated into the cytoplasm. However, there is an additional intralysosomal accumulation of indigestible residues and a point is finally reached at which the secondary lysosome is so filled with residual matter that it is no longer able to accumulate material for digestion. This stage is referred to as the residual body or telolysosome.[3] A concomitant decrease in enzymatic activity has been shown to occur along with the progressive accumulation of indigestible residues during the formation of the residual body. When no further enzymatic activity is demonstrable, the organelle is then referred to as a postlysosome. The residual body may remain within the cytoplasm as a nonfunctional postlysosome or extrude its contents from the cell by the process of exocytosis.[3]

Heterophagy is the method by which cells ingest and utilize exogenous substrates. Substrates enter the cell by the processes of phagocytosis, pinocytosis and micropinocytosis, collectively termed endocytosis. During endocytosis, foreign materials are engulfed by the plasma membrane through invagination and subsequent vesiclization.[3] The resultant substrate vacuole is variously referred to as a prelysosome, phagosome, or heterophagosome.[3,7] The heterophagosome fuses

with a primary or secondary lysosome to form a digestive vacuole in which the ingested substrate is subjected to the action of lysosomal enzymes.[3] The digestive and egestive processes then proceed in a fashion similar to that in autophagy.

Lysosomal Enzymes

Lysosomes are endowed with a wide variety of hydrolytic enzymes which suitably equip them for their functions in the digestion of intracellular and extracellular substrates. Barrett[8] has reviewed over fifty enzymes which are thought to be involved with lysosomal activity. These include various carboxyl ester and thiolester hydrolases, phosphatases, sulphuric esterases, glycosidases and proteases. Some of these enzymes are limited entirely to lysosomes, but many are also found in other intracellular organelles or have uncertain intracellular distributions. Nearly all have been found to have acid pH optima, although there are a few exceptions to this general rule. The activators of angiotensinogen, plasminogen and kininogen are active at neutral pH or above, and naphthylamidase, aspartylglucosylaminase, and dipeptidase have alkaline pH optima.

Although lysosomal enzymes are a functionally heterogenous group, features other than pH optima suggest homologies. Barrett[8] has hypothesized that these similarities may reflect evolutionary adaptations necessary for enzymatic activity in the specialized digestive vacuolar system. The lysosomal enzymes all have been found to be highly resistant

to autolysis,[9] a characteristic which is clearly of adaptative value. Furthermore, there do appear to be some structural similarities between those enzymes which have been purified sufficiently for meaningful analysis. The major similarity is the presence of carbohydrate. Thus, acid phosphatase, beta-glucuronidase, deoxyribonuclease II, hyaluronidase, beta-acetylglucosaminidase, cathepsin C and cathepsin D have all been found to be glycoproteins.[8]

Cathepsin D is the major lysosomal acid hydrolytic proteinase of many animal tissues and it has been shown to have a distinctly lysosomal distribution.[10,11] The molecular weight of the enzyme from various tissue sources has been estimated from 35,000 - 60,000[12,13] and amino acid analysis has demonstrated glycine to be its most abundant residue.[14] The active catalytic center of cathepsin D is thought to consist of two carboxyl groups, one of which is ionized. Barrett presumes these to be carboxyl groups of glutamic and aspartic acid.[8] The enzyme is known to act on several natural and synthetic substrates, including hemoglobin, globin, serum albumin, thyroglobulin, myosin, oxidized bovine pancreatic ribonuclease A, gelatin, casein, insulin and chondromucoprotein.[8] The enzyme acts by catalyzing the introduction of water at specific peptide bonds of its protein substrates. It is primarily an endopeptidase and as such acts on the interior peptide bonds of proteins.[15]

Cathepsin D demonstrates a characteristic acid pH optimum and its greatest activity has been shown to be against

hemoglobin at pH 3.0 - 3.5. The enzyme is irreversibly inactivated at pH 2.0 and demonstrates gradually decreasing activity against hemoglobin at pH values greater than 3.5. At pH 7.0, the enzyme is relatively inactive against hemoglobin.[16,17] With other proteins such as serum albumin and chondromucoprotein, optimum activity has been found to be at pH 4.0 - 5.0.[8]

Multiple isoenzymes of cathepsin D have now been reported in various tissues. Press et al[17] demonstrated ten different forms of the enzyme from bovine spleen which differed in charge at pH 5.5 and 8.4. Woessner[18] demonstrated four different isoenzymes from bovine uterus, and Barrett[19] resolved three major forms each from the liver of chicken and man. The various isoenzymes have all been found to have identical substrate specificities and pH dependence curves.

The Lysosomal Theory of Shock

It is apparent that the ability of lysosomal enzymes to perform physiologic functions is dependent on the structural integrity of lysosomal membranes. Any mechanism which sufficiently alters membrane structure would result in lysosomal enzyme release. Once freed into the cytoplasm or intercellular space, these hydrolytic enzymes might initiate deleterious changes. The ability of lysosomal enzymes to produce pathologic alterations in a variety of normal tissues has now been repeatedly demonstrated.[20,21,22,23,24,25,26]

In hypoprofusion states, ischemia results in hypoxia of various cells and tissues. Hypoxia has been shown

to induce labilization of lysosomal membranes and lysosomal enzyme release.[9,27,28] Furthermore, cellular hypoxia increases anaerobic glycolysis and results in a metabolic acidosis. Since lysosomal enzymes are most active in the acid pH range, it is not unreasonable to assume that the solubilization and activation of these enzymes would be accelerated by the accompanying metabolic acidosis. It has been postulated that in shock, the activation of these lysosomal hydrolytic enzymes initiates cellular autolysis, cell death and local tissue degeneration. Following their introduction into the systemic circulation these enzymes, or products of their action, may cause adverse effects to distant tissues, thus leading to a propagation of the shock syndrome, and a state of irreversibility.[24,29,30]

A large body of suggestive evidence has now accumulated which implicates lysosomal hydrolytic enzymes in the pathogenesis of shock. Numerous investigators have demonstrated increased levels of circulating lysosomal enzymes in a variety of shock states, including hemorrhagic shock,[24,31-36] bacterial endotoxin shock,[29,32,37,38] traumatic (drum) shock,[29,39] and splanchnic ischemic shock.[40] Janoff et al[29] demonstrated the release of beta-glucuronidase and cathepsins from the lysosomal fractions of liver obtained from animals subjected to traumatic and bacterial endotoxin shock. They further demonstrated elevations in plasma acid phosphatase and beta-glucuronidase during these forms of shock and found that these enzymes were released into the circulation in a

predictable manner. Hepatic lysosomal homogenates from shocked animals also revealed an increase in the ratio of enzymatic activity found in soluble versus sedimentable fractions, which suggested an in vivo loss of lysosomal membrane integrity. In vitro studies were confirmatory of increased hepatic lysosomal fragility. When hepatic lysosomal suspensions from shocked animals were incubated at 37° or subjected to mercury arc irradiation, a greater release of cathepsins occurred than was the case with suspensions obtained from unshocked controls.

Reich et al[35] studied plasma catheptic activity in cats subjected to hemorrhagic shock and reported a significant relationship between plasma cathepsin activity and mortality. Animals which developed greater than twice the normal plasma cathepsin level during shock had a 50 per cent higher mortality rate than animals which failed to develop twice normal levels. Courtice et al[36] reconfirmed the appearance of acid phosphatase, beta-glucuronidase and cathepsin D in the plasma of animals during hemorrhagic shock. These investigators further documented increases in acid ribonuclease and N-acetyl-B-D-glucosaminidase during hemorrhagic shock and demonstrated a parallelism between plasma lysosomal enzyme levels and the duration of shock.

Another major category of evidence for the role of lysosomes in shock has been the demonstration of multiple deleterious effects resulting from the infusion of lysosomal constitutents into experimental animals. Gazzaniga and

O'Connor[41] observed the effects of intravenous infusions of autologous kidney lysosomal enzyme extracts in intact dogs. In their series, infusions of these extracts resulted in hypotension, altered blood coagulation, granulocytopenia, hepatic congestion and changes in the viability of the gastrointestinal tract, including gastrointestinal bleeding and infarctions of the mesentery, omentum and bowel wall. Both sedimentable and soluble lysosomal-rich fractions were utilized for infusion. These preparations were obtained by homogenization of dog kidney followed by two sequential ultracentrifugations at 15,000 x g.

Glenn et al[42] subjected dogs to infusions of hepatic lysosomal extracts obtained by techniques similar to those of Gazzaniga and O'Connor. Infusion into normal dogs resulted in significant decreases in mean arterial blood pressure, cardiac output and superior mesenteric artery blood flow. These changes were also noted to parallel increases in plasma cathepsin D activities. A second group of dogs was subjected to exclusion of the major portion of the reticuloendothelial system by splenectomy and portocaval shunt prior to lysosomal extract infusion studies. This preparation bypasses the natural defenses of the reticuloendothelial system and allowed greater expression of lysosomal hydrolase activity. In this series, the hemodynamic effects of lysosomal extract infusions were more pronounced and a significant (67 per cent) mortality rate was demonstrated. Furthermore, perfusion of isolated cat heart preparations with these extracts was found to produce significant decreases in cardiac contractile force

and coronary artery blood flow. In order to determine whether this negative inotropic effect was due solely to coronary artery vasoconstriction or to a direct cardiodepressant effect, lysosomal extracts were added to isolated cat papillary muscles. The addition of these extracts resulted in marked decreases in the developed tension of these muscles, indicating a direct cardiodepressant action of lysosomal hydrolases. These authors therefore concluded that lysosomal enzymes contribute to the pathophysiology of circulatory shock by a direct depressant action on the heart and by a marked increase in peripheral vascular resistance. .

In addition to their hemodynamic effects, direct vasculotonic effects of lysosomal enzymes have been demonstrated. Sutherland et al[24] studied the myocardial vessels of isolated canine hearts perfused with lysosomal extracts. Histologic examination of these vessels repeatedly demonstrated lesions consisting of endothelial proliferation with partial occlusion of the vessels and fragmentation of basement membranes. Janoff et al[22] observed multiple pathologic changes in the microcirculation of rat and rabbit mesentery following the direct application of lysosomal extracts. Lysosomal constituents have also been shown to injure cutaneous vessels of rabbits causing increases in capillary permeability and the development of Arthus- and Swartzman-like lesions.[21,43]

Of the numerous lysosomal enzymes, the acid proteases, and in particular the cathepsins, have been implicated as the agents most responsible for deleterious effects in

shock. In addition to the previously mentioned lines of evidence, several investigators have reported that protease-cathepsin inhibitors afford protection in various experimental shock states. Back et al[44] studied the effects of these pharmacologic agents in shock caused by trauma, burn and anaphylaxis. The protease inhibitor trasylol, and to a lesser extent epsilon amino caproic acid, significantly reduced mortality in these shock systems during their acute phases. Both agents also caused marked reductions in plasma catheptic activity. Trasylol demonstrated a significant dose-response relationship in all these forms of shock and caused the greatest decreases in plasma cathepsin levels. Other investigators have confirmed the enhanced survival of experimental animals in shock following trasylol administration.[45,46] Furthermore, Smith et al[47] demonstrated that when administered before the induction of hemorrhagic shock, trasylol maintained cardiac output at higher levels and total peripheral resistance at lower levels than expected.

A recent immunologic study has also suggested a detrimental role for lysosomal enzymes in shock. Jones and Wangensteen[48] pretreated rabbits with a specific anticathepsin D antiserum prior to the induction of hemorrhagic shock. These passively immunized animals demonstrated a 47 per cent increase in survival as compared to non-immunized control animals. This finding suggests that the removal of cathepsin D by formation of antigen-antibody complexes prevents the deleterious effects of this acid protease in circulatory shock.

Despite all of the accumulated experimental evidence, the role of lysosomal enzymes in shock remains controversial. Neither the chronological sequence of lysosomal disruption in shock nor its significance in terms of cell destruction has been adequately defined. Whereas some biochemical and histochemical studies have demonstrated damage to lysosomal membranes early in the course of experimental shock, others have reported this as a late finding. Sutherland et al[24] presented histochemical evidence to show that lysosomal disruption and intracellular scatter of acid phosphatase occurs within two hours after the onset of severe hemorrhagic shock. In the intestinal epithelium of dogs subjected to profound hypotension, the intracellular dispersal of lysosomal acid phosphatase was found to precede and signal early necrotic changes. Kerr,[49] however, reported that following ischemic hypoxia of the liver, marked changes were evident in those cells destined to undergo coagulative necrosis before any abnormality could be detected in the lysosome by histochemical methods. Furthermore, the appearance of lysosomal hydrolases in the circulation during shock cannot be taken as sufficient evidence that they are the primary agents of destructive effects. Certainly their appearance could as easily represent an epiphenomenon occurring secondary to other intracellular alterations in shock states, or their action may be contributory rather than primary in the development of irreversible changes during shock.

The various biochemical, histologic, pharmacologic and immunologic studies offer highly suggestive but indirect

evidence for an important role of lysosomal proteases in shock. More direct evidence has resulted from the demonstration of multiple damaging effects following the infusion of lysosomal preparations into experimental animals. These studies have reproduced some of the pathophysiologic changes that characterize the shock state. In recent years, methods have been devised for the isolation and purification of cathepsin D. It is the purpose of the present investigation to adopt a method for obtaining purified cathepsin D and to directly evaluate the role of this enzyme in circulatory shock. Experimental models were designed to assess the effects of massive doses of this enzyme in normal animals and in animals subjected to either mild or severe hemorrhagic shock. Further studies were undertaken to observe the direct effects of cathepsin D upon the heart and microcirculation.

II. EXPERIMENTAL METHODS AND MATERIALS

Cathepsin D Purification

The method of Barrett,[50,51] with modifications, was used for cathepsin D isolation. Commercially obtained, young, rabbit liver (2.0 kg) was homogenized in 4.0 liters of 1 per cent saline solution containing 2 per cent 1-butanol. Calcium chloride dihydrate (88 gm) was dissolved in the homogenate and 600 cc of 1 M dipotassium hydrogen phosphate were added while stirring. The pH of the homogenate (approximately 5.5-6.0) was adjusted to 7.0 by the addition of 2 M tris base HCl buffer (Sigma 1503 tris base, 242 gm, dissolved in approximately 850 cc deionized water, adjusted to pH 9 by the addition of 6 M HCl and diluted to a liter of 2 M solution by further addition of deionized water). The homogenate was then centrifuged at 1500 x g in a Sorvall RC2B ultracentrifuge for 30 minutes at 15° C. The resultant precipitate was discarded. The supernatant was adjusted to pH 3.6 with 5 M sodium formate-formic acid buffer of pH 3.0 and incubated for 12 hours at 37° C. The supernate was cooled to 0° C and 0.9 volumes of anhydrous acetone (dried with anhydrous sodium sulfate) were added over a 10-minute interval. The preparation was centrifuged at 1500 x g for 15 minutes at 0° C, the precipitate was discarded, and a second volume of acetone equal to the first was added to the supernate. The subsequent precipitate was collected by vacuum filtration through a Buchner funnel with Whatman #1 filter paper in the presence

of celite analytical filter aid. The filtrate was discarded and the precipitate redispersed in a minimum volume of 50 mm EDTA trisodium salt in a buffer solution at pH 8.0 of 50 mm tris base-tris HCl (made by the addition of 17.9 gm EDTA trisodium salt to 2.65 gm tris base and 4.44 gm tris HCl and diluted to 1 liter). The preparation was dialyzed over a 24-hour period against two changes of the EDTA tris buffer solution and then redialyzed for 24 hours against a 2 mm tris HCl buffer solution of pH 9.0 until the pH of the preparation was above 7.5. The preparation was then filtered as before, the filtrate was preserved and the precipitate discarded. Approximately 300 cc of enzyme preparation was available at this stage. The preparation was applied to a 200 cm^3 column of Whatman #DE52 DEAE cellulose equilibrated with tris HCl buffer solution (14.4 mm tris base brought to pH 7.8 with 10 mm HCl). The column was eluted with two liters of 0.2 M sodium chloride in the tris HCl buffer along a linear gradient. The rate of elution varied from 75 to 100 cc/hour. Ten cc samples were collected using an Instrument Specialties Model #400 volumeter and Model #328 fraction collector and 1 µl sample fractions were assayed for cathepsin D activity. Those samples with high (>20 units/cc) activity were pooled and dialyzed for 12 hours against 30 mm acetic acid-30 mm sodium acetate buffer of pH 4.75. The dialysate was applied to a volume of Whatman #CM52 DM cellulose, bedded to 15 cm^3, and equilibrated with 30 mm acetic-acid-30 mm sodium acetate buffer, pH 4.75. The column was eluted with an appropriate

amount of 50 mm acetic acid-200 mm sodium acetate (2.9 cc glacial acetic acid and 27.2 gm sodium acetate in one liter deionized water) and 3 cc fractions were collected. Fractions with high (>20 units/cc) cathepsin D activity were pooled and stored at 0° C. Elution was then continued until fractions were obtained which demonstrated no cathepsin D activity. These fractions were pooled and stored at 0° C for use as control solution.

Biochemical Determinations

Plasma cathepsin D activity was assayed using a combination of the methods of Barrett[50] and Anson.[52] The assay is based on the determination of acid-soluble peptides released from hemoglobin substrate by enzyme sample. A mixture of 0.5 cc plasma sample, 0.25 cc 8 per cent hemoglobin substrate (Sigma H-2500 type I bovine hemoglobin) and 0.25 cc 1 M formate buffer, pH 3.0 was incubated in a Dubnoff metabolic shaker at 37° for 60 minutes. Proteolytic action was stopped by the addition of 0.3 M trichloroacetic acid (5 cc) and the mixture allowed to stand at room temperature for five minutes. The preparation was then filtered through Whatman filter paper #1 and 2 cc of the clarified filtrate was added to 4 cc 1 N NaOH. After 10 minutes at room temperature 2/3 N folin ciocalteau phenol reagent was added and mixed immediately. Thirty minutes thereafter, optical density was determined at 700 mμ on a Coleman Jr. spectrophotometer. Blanks were obtained by the addition of plasma sample to

hemoglobin substrate buffer after incubation followed by mixing with trichloroacetic acid. Enzymatic activity is determined by the difference in optical density between enzyme sample and blank at 700 mμ. Cathepsin D specific activity was expressed as milliequivalents of tyrosine x 10^{-6} produced per milliliter sample per milligram protein per hour at 37°. Relative activity was defined as milliequivalents tyrosine x 10^{-4} produced per milliliter sample per hour at 37°.

Protein was determined by the method of Lowry et al[53] for the purification preparations and by the biuret technique for plasma samples. $[Na^+]$ and $[K^+]$ were determined by a flame spectrophotometer. $[Cl^-]$ was analyzed by a chlorimeter. Other biochemical parameters were determined by Biomedical Laboratories, Inc.*

Intact Animal Preparation

New Zealand white rabbits, weighing between 3 and 3.5 kg were anesthetized by the administration of acepromazine (0.3 mg IV) and sodium pentobarbitol (20 mg/kg IV). The left carotid artery was cannulated with a 14 F polyethylene catheter. Mean arterial blood pressure (MABP) and heart rate were monitored using a Statham P-23 Db pressure transducer and recorded on a Hewlett-Packard 8805 B carrier amplifier calibrated with a pressure range from 0 to 100 mm Hg. A rectal probe connected to a tele-thermometer was inserted for monitoring core body temperature. Initial MABP, heart rate and temperature were recorded and a 3 cc blood sample

*Biomedical Laboratories, Inc., Richmond, Virginia.

was obtained for cathepsin D assay. The purified cathepsin D preparation was given intravenously over a five-minute interval. The volume for infusion was calculated, on the basis of animal weight, to give serum cathepsin D levels equal to or greater than that found in severe hemorrhagic shock. Over the ensuing two-hour experimental period, MABP, heart rate and temperature were recorded and 3 cc blood samples were obtained for cathepsin D assay at 15-minute intervals. Three cubic centimeters of heparinized saline solution was infused after each blood sampling in order to maintain fluid balance. At the termination of the experiment, the arterial cannula was removed, the animal was returned to its cage, and its condition observed at 8, 16, and 24 hours following infusion.

Hemorrhagic Shock Preparations

Twenty-four New Zealand white rabbits were prepared and monitored in a fashion similar to that for the intact animal study. Initial MABP, heart rate and temperature were recorded and a 3 cc blood sample was obtained for pH and cathepsin D assay. Arterial blood was then rapidly drained into heparinized syringes. In approximately five minutes a mean pressure of 40 mm Hg was reached and this level was carefully maintained for 60 minutes. Following hemorrhage, half the animals were infused over a five-minute interval with an appropriate quantity (200 units relative activity/kg body weight) of purified cathepsin D and half with equal volumes of control solution. During the following one hour

the animals were allowed to equilibrate, after which time the remaining shed blood was reinfused. MABP, heart rate and temperature were monitored and 3 cc blood samples were obtained for cathepsin D assay every 15 minutes during the periods of hemorrhage and equilibration and for one hour after blood reinfusion. Following hemorrhage, 3 cc of heparinized saline was administered after each blood sampling. Arterial blood pH was determined initially and after each hour of the total three-hour experiment. At the termination of the experiment, the arterial cannula was removed, the animal was returned to its cage, and its condition was observed at eight-hour intervals for twenty-four hours following infusion.

An additional 30 rabbits were subjected to all of the above procedures except that these animals were bled into severe shock with the MABP held at 25 mm Hg during the period of hemorrhage. Fifteen animals received the purified cathepsin D preparation and the remaining 15 served as controls. Plasma beta-glucuronidase activity was also measured initially and at one-hour intervals throughout the experimental period.

Microcirculation Studies

The effect of cathepsin D upon vascular integrity was assessed by the Evans blue dye technique.[21,23] Two intradermal abdominal injections of purified cathepsin D at a dosage of ten units relative activity/injection were given into the clipped abdominal skin of rabbits anesthetized with sodium pentobarbitol. For controls, each animal received

two additional intradermal injections each of the prepared control solution (0.15 cc/injection), bacteriostatic saline (0.15 cc/injection) and histamine phosphate (3 mg/injection). One hour following preparation of the skin, Evans blue dye at a dosage of 0.3 cc/kg of a 5% saline solution was administered intravenously. Fifteen minutes after the dye injection, the animals were sacrificed and the diameter of any locally extravasated dye was measured on the inner surface of the skin. The diameter and intensity of "bluing" around the injection site which results from locally leaked dye is an indication of alterations in vascular permeability. Bluing intensity was graded from 0 to ++++. Eight animals were studied and the cathepsin D used for injection was varied between the products of three purification procedures.

In order to more fully evaluate the effects of cathepsin D upon peripheral vascular homeostasis, its action on the microcirculation of rabbit mesentery in the living state was observed. The method of Zwiefach and Metz[54] was used for the preparation of rabbit mesentery maintained under physiologic conditions. Rabbits, lightly anesthetized with sodium pentobarbitol (15 mg/kg), were subjected to laparotomy and gentle exteriorization of a portion of the small bowel mesentery. The mesentery was loosely draped over a specially prepared plexiglass stage of a Bausch and Lomb binocular light microscope and immediately irrigated by drip through a 19 gauge needle with Ringer gelatin solution rigidly maintained at 37° C by a Tamson circulating thermostatic bath.

Adequate microscopic observation of the mesenteric microcirculation was obtained by magnification with a 10 x ocular lens in combination with a 10 x objective or 40 x water immersion lens. Following visual checks on the normal appearance of the circulation, purified cathepsin D was applied topically and allowed to remain on the tissue for selected time intervals. The quantities of enzyme preparation applied were varied from 1 to 3 cc of preparations containing 59.9 and 48.8 enzyme units/cc. Following application, the mesenteric vessels were observed for periods up to 45 minutes for changes in blood flow, capillary permeability and leukocyte immigration.

Myocardial Muscle Assay

Purified cathepsin D preparations were assessed for inotropic activity on isolated rabbit myocardial muscles by the method of Lefer.[55] New Zealand white rabbits weighing 3.5 to 4.0 kg were anesthetized with intravenous sodium pentobarbitol (25 mg/kg). A cardiectomy was rapidly performed through a median sternotomy under positive pressure ventilation and the heart was immediately placed in a continuously oxygenated bath of modified Krebs-Henseleit buffer solution at room temperature. Two left ventricular muscle slips, averaging 8 to 10 mm in length and 1 mm in diameter, were dissected free and secured at each end with 4-0 silk ligatures while submerged in the buffer bath. Each muscle was then vertically mounted by the ligatures in the inner chamber of a 10 cc double jacketed constant temperature chamber

containing Krebs-Henseleit buffer. The concentration of the Krebs-Henseleit buffer, in millimoles/liter, was: NaCl, 118; KCl, 4.75; $CaCl_2 \cdot 2H_2O$, 2.54; KH_2PO_4, 1.19; $MgSO_4 \cdot 7H_2O$, 1.19; $NaHCO_3$, 12.50; and glucose, 10.00. The solution was equilibrated with 95% O_2 and 5% CO_2 to a pH of 7.3. Chamber temperature was maintained at 37° C by a Tamson circulating thermostatic bath and the muscle chamber was aerated continuously with 95% O_2 and 5% CO_2.

The myocardial muscles were electrically stimulated by platinum disk electrodes 8.7 ± 0.2 mm in diameter fixed on both sides of the mounted muscle at an interelectrode distance of 6.0 ± 0.5 mm. Stimuli consisted of 20 msec pulses of alternating current of 3.75 to 4.00 volts peak applied at a rate of 1 pulse/sec. The alternating current pulse stimulator consisted of a 44 volt Grass SM6 stimulator equipped with variable frequency and duration controls connected in line to a variable 0 to 10 volt step-down transformer. Muscle tension was registered on a Grass FT032 force displacement transducer and recorded on a Sanborn Model 60-1300 Twin Viso equipped with Sanborn Model 64-500B strain gauge amplifiers. The strain gauge amplifiers were balanced and calibrated daily with a 1 gram standard weight. A length-tension relationship was determined for each muscle, and the muscle tension was set to a level just below that at which electrical stimulation produced maximal developed tension during isometric contractions. This was approximately 1 gm for a muscle of 1 mm diameter.

After the muscles developed a constant resting tension and contractile force, the Krebs-Henseleit buffer was removed by aspiration and replaced with either cathepsin D or control solution. Cathepsin D was tested at increasing concentrations up to 40 units relative activity/cc. Following each assay for inotropic activity, the muscle bath was washed out and renewed with fresh Krebs-Henseleit solution prewarmed to 37° C, and the muscle allowed to return to its resting tension. Inotropic activity was measured as the per cent change in developed tension of the sample bathing the muscle compared to activity in the Krebs-Henseleit buffer standard.

Statistical Analysis

All reported values, unless otherwise indicated, are expressed as the mean ± the standard error of the mean (SEM). Analysis for statistical significance was made by the unpaired student "t" test and, where appropriate, by the paired "t" test. Probability values less than 0.05 were considered statistically significant.

III RESULTS

Cathepsin D Purification

Using a total of 16 kg rabbit livers, 994 cc of purified enzyme extract containing a mean 44.9 ± 4.9 enzyme units/cc were produced (table 1). High yields, averaging 44% of the initial homogenate enzymatic activity, were obtained. Overall, the procedure resulted in an approximate 203-fold increase in purity but purification, particularly following DEAE cellulose column chromatography, was less than expected. The final preparations averaged 121.8 ± 22.9 enzyme units/mg protein with a mean 0.56 ± 0.23 mg/cc total protein.

Assay for enzymes other than cathepsin D was performed on the purified enzyme extracts. The lysosomal enzymes acid phosphatase and beta-glucuronidase were not detectable in any of the final preparations. Other liver enzymes were present in small quantities (table 2). As expected, sodium concentrations were high, but other electrolytes were absent with the exception of trace amounts of calcium. Cholesterol was found to be the other non-protein contaminant most consistently present. Analysis of the prepared control solutions failed to reveal any catheptic activity and demonstrated a close similarity to the cathepsin D preparations in terms of contamination. The pH of both preparations averaged 5.2.

Infusion Studies

Normal plasma cathepsin D was measured in 58 healthy New Zealand white rabbits and found to be 1.65 ± 0.19 units/mg protein (0.90 ± 0.10 unit/cc). Following infusion of the purified cathepsin D preparation in seven normal rabbits, enzyme activity increased dramatically. At 15 minutes, plasma cathepsin D activity had increased 467% to 38.20 ± 4.38 units/mg protein (figure 2). Over the ensuing 105 minutes a gradual but progressive fall in plasma cathepsin D activity occurred, probably as a result of clearance of the enzyme by the reticuloendothelial system. However, at two hours following infusion, plasma cathepsin D remained significantly elevated over initial levels ($p < .005$) at 20.10 ± 3.95 enzyme units/mg protein which is in the upper range observed in shock states.[36]

The hemodynamic and temperature responses to the infusion of cathepsin D in normal rabbits are summarized in table 3. No statistically significant change was noted in either MABP or heart rate during the entire two-hour experiment. A significant fall in temperature ($p < .005$) was observed at 60 minutes following infusion and persisted throughout the remaining experimental period. No animal died following infusion and all animals were judged to be in good health 24 hours following infusion.

Figure 3 summarizes the variations in plasma cathepsin D activities in rabbits subjected to one hour of

mild (MABP = 40 mm Hg) hemorrhagic shock prior to the infusion of either purified cathepsin D or control solution. Fifteen minutes after the initiation of hemorrhage, plasma cathepsin D demonstrated a mean 41% increase in activity over preshock levels. After one hour of shock, activities increased to 15.56 $\pm$ 3.02 units/mg protein in the cathepsin D infusion group and 21.80 $\pm$ 4.83 units/mg protein in the controlls, representing respective increases of 750% ($p < .001$) and 1183% ($p < .001$). Fifteen minutes following infusion of the purified enzyme extract, plasma cathepsin D increased 3812% over initial levels to a peak 71.59 $\pm$ 4.40 units/mg protein. In the control animals, levels gradually increased 1972% to a maximum 35.23 $\pm$ 7.04 units/mg protein at fifteen minutes after infusion of the prepared control solution. Rabbits which received the purified cathepsin D maintained significantly higher plasma cathepsin D activities following infusion as compared to the controls. At one hour after the reinfusion of shed blood, cathepsin D remained significantly elevated in these animals at 30.68 $\pm$ 3.61 units/mg protein versus 14.73 $\pm$ 2.87 units/mg protein in the control rabbits ($p < .005$).

The hemodynamic, temperature and arterial blood pH responses of the experimental and control animals are summarized in table 4. No statistically significant differences between the cathepsin D and control solution infused rabbits were observed in these parameters during the entire experimental period. The MABP was approximately 40 mm Hg after one

hour of hemorrhage and the mean maximal amount of shed blood was identical (24 ± 3 cc/kg) in both groups. Each group demonstrated mild elevations in MABP after infusion of purified cathepsin D or control solution as a response to volume expansion, and both groups experienced similar responses to the reinfusion of blood. Fifteen minutes after blood reinfusion the MABP of cathepsin D-infused rabbits increased 139% to 85.2 ± 3.7 mm Hg, while that of controls increased 128% to 85.1 ± 8.6 mm Hg. Over the following 45 minutes both groups maintained approximately the same MABP. The heart rate and temperature responses of both groups were also highly analogous and alterations in these parameters were as predictable as MABP. Although intact animals infused with cathepsin D demonstrated significant depression in core body temperature, no significant differences in temperature were observed between rabbits receiving cathepsin D and those receiving control solution. Rabbits infused with the purified enzyme preparation developed an average maximal fall of 1.1° C in temperature with peak depressions occurring 45 minutes after blood reinfusion. Core body temperature of controls demonstrated a mean maximal depression of 0.6° C with peak depressions 30 minutes after blood reinfusion. Both groups also experienced nearly identical arterial blood pH alterations. Hemorrhage was followed by the development of a mild acidosis which gradually resolved during the remaining experimental period.

The mortality rate was 33% in rabbits infused with cathepsin D and 17% in controls. The difference in these rates is not statistically significant.

Animals subjected to severe (MABP = 25 mm Hg) hemorrhagic shock demonstrated profound increases in plasma cathepsin D activities (figure 4). Within 15 minutes following the induction of shock, enzyme levels increased 91% to 3.15 ± 0.69 units/mg protein ($p < 0.02$) and 36% to 2.95 ± 0.42 units/mg protein in the experimental and control groups, respectively. One hour after the induction of shock, activities were markedly elevated and demonstrated a 2387% increase to 41.03 ± 3.35 units/mg protein in the experimental group and a 1588% increase to 36.65 ± 3.47 units/mg protein in the controls. The difference in plasma cathepsin D activities between groups after one hour of shock was not significant and no differences in MABP, heart rate or core body temperature were observed during hemorrhage. The mean maximal amount of blood shed during hemorrhage was also similar. Rabbits which received purified cathepsin D had a mean 33.5 ± 0.9 cc/kg blood removed during hemorrhage. A mean 34.0 ± 1.0 cc/kg blood were shed from controls.

Those animals infused with purified cathepsin D demonstrated a total 5870% ($p < 0.001$) increase in plasma cathepsin D activity which peaked at 98.51 ± 5.17 units/mg protein 15 minutes after infusion. During the following period of equilibration, enzyme levels gradually decreased but a sharp depression occurred after the reinfusion of blood, probably as a result of hemodilution. After blood reinfusion, plasma cathepsin D levels again demonstrated a gradual progressive decrease in activity. However, one hour after the

reinfusion of blood, plasma activities remained significantly elevated at 48.92 ± 1.97 units/mg protein ($p < .001$).

The control rabbits experienced a continual rise in plasma cathepsin D activity following the infusion of control solution. Activities peaked 60 minutes after infusion at 47.39 ± 3.96 units/mg protein. Following blood replacement a sharp decline in plasma activity occurred which was similar to that in the experimental group. Thereafter, activities declined progressively although a small but insignificant elevation occurred at the termination of the experiment. Plasma cathepsin D activity was significantly elevated in rabbits which received the purified enzyme preparation as compared to the control rabbits at all times following infusion.

Table 5 summarizes the hemodynamic, temperature and arterial blood pH responses of rabbits infused with either purified cathepsin D or control solution following one hour of severe hypotension. MABP, heart rate and core body temperature did not vary significantly between groups throughout the three-hour experiment. Elevations in MABP occurred predictably in both groups as a result of volume expansion following the infusion of the cathepsin D preparation or control solution and after blood reinfusion. Both groups also maintained close similarities in MABP and heart rate after the reinfusion of blood. One hour following blood reinfusion, rabbits which received the cathepsin D preparation demonstrated a MABP of 71.4 ± 7.1 mm Hg and heart rate of 255 ± 6 beats/-

minute. Controls at this same time exhibited a MABP of 63.5 ± 4.7 mm Hg and heart rate of 243 ± 8 beats/minute. Similar depressions in core body temperature occurred in both groups during t e experiment. The temperature of rabbits infused with cathepsin D decreased from an initial 38.2 ± 0.1° C to 36.9 ± 0.2° C 15 minutes after blood reinfusion. Control rabbits also experienced maximal depression in core body temperature 15 minutes after blood reinfusion and demonstrated a decrease from an initial 38.2 ± 0.1° C to 36.6 ± 0.2° C. Temperature depressions probably represented normal physiologic responses to hemorrhagic shock and to the administration of room temperature solutions and blood. Both groups demonstrated significant but similar changes in arterial blood pH. The experimental animals experienced a mean 0.28 ± 0.02 decrease ($p < .001$) in arterial pH from an initial pH of 7.48 ± 0.01 to 7.20 ± 0.03, 60 minutes after the induction of shock. Over the same interval, control rabbits exhibited a mean 0.27 ± 0.02 decrease ($p < .001$) in arterial pH from an initial pH of 7.50 ± 0.01 to 7.23 ± 0.03. Following the infusion of cathepsin D or control solution, acidosis persisted, but after blood reinfusion, arterial pH demonstrated a return to normal values. Sixty minutes following the reinfusion of blood, arterial pH was 7.41 ± 0.05 and 7.38 ± 0.04 in rabbits which received cathepsin D or control solution, respectively.

Plasma levels of beta-glucuronidase demonstrated similar patterns in rabbits which received cathepsin D and rabbits infused with control solution (figure 5). Both groups

exhibited increases in plasma activity of this enzyme during hemorrhage and after infusion, and declines in activity after blood reinfusion. No statistically significant effect on plasma beta-glucuronidase activity was demonstrated following the infusion of high activities of cathepsin D. The initial levels of plasma beta-glucuronidase in rabbits infused with cathepsin D was 46.0 ± 4.1 units. This increased to 164.0 ± 19.7 units following 60 minutes of shock and to 328.9 ± 39.4 units one hour after the infusion of cathepsin D. One hour following blood reinfusion, activity declined to 255.3 ± 42.0 units. Similarly, plasma beta-glucuronidase of control rabbits was 39.1 ± 3.3 units initially, 143.8 ± 26.2 units after one hour of shock, 256.2 ± 44.4 units one hour following infusion of control solution and 218.6 ± 38.3 units one hour after the reinfusion of shed blood.

Rabbits infused with purified cathepsin D demonstrated a greater but statistically insignificant mortality rate than controls (67% vs. 47%). Furthermore, there was no significant difference in the mean survival time between groups. The mean survival time for animals which received cathepsin D was 468 ± 137 minutes (measured from the time of cathepsin D infusion), whereas that for control rabbits was 622 ± 237 minutes. Four of 15 rabbits infused with cathepsin D and eight of 15 controls survived more than 24 hours following infusion.

Microcirculation Studies

The cutaneous vascular responses to intradermal injections of purified cathepsin D and appropriate controls are summarized in table 6. Histamine phosphate was included in this study as a known positive control, bacteriostatic saline as a known negative control. There was local extravasation of Evans blue dye at all histamine injection sites, at 11 of 16 cathepsin D injection sites, five of 16 prepared control solution injection sites, and two of 16 saline sites. Histamine caused the greatest response, both in terms of diameter and intensity of bluing and the average zone of dye leakage about histamine injections was significantly greater than that surrounding cathepsin D injections. However, cathepsin D caused lesions larger than both the prepared control solution and saline. The average diameter of dye extravasation surrounding injections of cathepsin D was 3.0 ± 0.7 mm as compared to 0.9 ± 0.4 mm surrounding the prepared control solution ($p < .02$) and 0.3 ± 0.2 mm around saline ($p < .001$). No difference in the intensity of bluing was observed between cathepsin D and control solution.

The topical application of purified cathepsin D on exteriorized rabbit small bowel mesentery failed to demonstrate any direct vasculotoxic effects. Mesenteric fields were observed which contained at least eight vessels consisting of various numbers of arterioles, precapillaries, capillaries and collecting venules. Blood stasis occurred in a

small percentage of these vessels immediately after the application of the cathepsin D preparation. Precapillaries were most frequently affected and responded by a gradual reduction in the normal retrograde ocillations of blood flow followed rapidly by the complete stasis of blood. However, the majority of vessels continued to function unaltered for periods up to 45 minutes after the application of cathepsin D. Altered vascular integrity secondary to the action of cathepsin D could not be confirmed in this study. There also was no evidence either of leukocyte emigration or leukocytic adherence to endothelial surfaces.

Myocardial Muscle Assay

Cathepsin D was assayed for inotropic activity at concentrations of 4, 8 and 40 units/cc. The 4 and 8 units/cc solutions were prepared by respective 1:10 and 1:5 dilutions of the 40 unit/cc sample in standard Krebs-Henseleit buffer. Several samples of each concentration were extensively assayed on ten different myocardial muscles. The results of these assays are summarized in table 7. Cathepsin D at maximal concentration produced marked positive inotropic effects for three minutes after addition to the isolated muscle and a small insignificant negative inotropic effect one minute thereafter. No significant differences were noted between the effects of cathepsin D and control solution at maximal concentrations. Cathepsin D and control solution at the dilute concentrations did not demonstrate the marked positive

inotropic effects of the maximally concentrated preparations and produced inotropic effects similar to one another.

IV DISCUSSION

Previous studies have demonstrated multiple deleterious effects following the infusion of lysosomal preparations into normal experimental animals. Refractory hypotension, myocardial depression and enhanced splanchnic vasoconstriction with pathologic alterations of the gastrointestinal tract have been produced most consistently. Glen et al[42] further observed that animals subjected to surgical removal of the major portion of the reticuloendothelial system demonstrated more profound cardiovascular effects which correlated with high plasma cathepsin D levels following the infusion of lysosomal preparations. These results have been interpreted by some investigators as being consistent with the hypothesis that release of lysosomal enzyme into the circulation may be responsible for the progressive deterioration of circulatory functions seen in the irreversible phase of shock.

In spite of these findings, several problems are inherent in previous infusion studies. Foremost is the fact that infusates were prepared by the techniques of tissue homogenization and differential ultracentrifugation. Since individual lysosomes vary widely in size and have sedimentation coefficients similar to other subcellular organelles,[56] isolation is difficult using these methods. These infusates contained innumerable enzymes derived from multiple subcellular sources and included mitochondria, microsomes and endoplasmic reticulum as well as lysosomes. Furthermore, the

preparations contained large quantities of nonenzymatic proteinaceous contaminants of cellular and intercellular origin. It is therefore difficult to ascribe the observed results directly to lysosomal enzymatic action or to products of their action. The present study deleted these extraneous factors so that the effects of a single lysosomal enzymatic species -- i.e., cathepsin D -- could be observed.

Barrett's method for the isolation and purification of cathepsin D was found to be reproducible and gave quantities of purified enzyme which were sufficient for physiologic study. Although Barrett reported higher increases in purification (870 vs. 203), our final preparations were sufficiently low in total protein content and contaminants so as to preclude interference with physiologic studies. None of the extracts contained particulate contaminants and the final preparations were highly clarified (figure 6). Purification could have been increased somewhat by pooling only those fractions of very high specific activity. However, this would have been at the expense of percentage yield and would have reduced the quantities of enzyme product available for study. The major criticism of the procedure, emphasized by Smith and Turk,[57] is that it is lengthy and may allow some proteolysis of cathepsin D by other enzymes present in the system during isolation.

The present investigations failed to demonstrate any hemodynamic effects of purified cathepsin D infusions in intact rabbits. MABP and heart rate remained stable throughout

the experiment. A significant depression in core body temperature was observed in all of the animals studied. This finding was unexpected and is difficult to explain since appropriate controls were not studied. Many undetected changes such as splanchnic vasoconstriction may have occurred which could depress body temperature, but the etiologic agent(s) of this effect are conjectural. Rabbits are known to respond to deep pentobarbitol anesthesia by progressive decreases in core body temperature,[54] and this is equally as likely an explanation as any direct effect caused by cathepsin D. No other untoward effects were observed and all animals survived the experiment.

The reticuloendothelial system has been shown to be in large part responsible for the clearance of circulating lysosomal enzymes. Lentz and Smith[58] demonstrated reduced levels of plasma acid phosphatase and beta-glucuronidase during hypotension in animals pretreated with reticuloendothelial stimulating agents. Zymosan, a complete cell wall extract and known stimulant of reticuloendothelial function, was found to give the smallest increases in plasma lysosomal hydrolase activity during shock. Surgically accomplished reticuloendothelial exclusion prolongs half-life of the enzyme and results in greater increases in plasma levels during infusion studies. Fredlund et al[59] documented prolonged hydrolase disappearance following portocaval shunt. In one case, the reestablishment of circulation resulted in the redevelopment of a nearly normal elimination rate. Glen et al[42]

subjected dogs to splenectomy as well as portocaval shunt prior to the infusion of unpurified lysosomal preparations. This resulted in a 170% increase in plasma cathepsin D levels over that of intact, infused animals, and the enzyme was found to be elevated over prolonged periods. In the present intact animal series, plasma cathepsin D increases were so massive (467%) that reticuloendothelial exclusion was deemed unnecessary. Even at two hours following infusion, enzyme levels were markedly increased (240%). Indeed, Glen et al did not achieve greater initial increases (400%) and at two hours, levels had fallen below those in the present series.

Few studies have been reported on the disappearance of lysosomal enzymes from plasma. Huggins[60] found that the half-life of acid phosphatase, following its intravenous administration to dogs, was approximately 40 minutes. Fredlund et al[59] studied the plasma disappearance of beta-glucuronidase and beta-galactosidase following the infusion of crude lysosomal extracts into pigs. These enzymes were found to follow an exponential rate of elimination regardless of the dose of infusate and both had half-life periods of approximately one hour. In the present study, the half-life of cathepsin D, by extrapolation, was found to be about 90 minutes in normal rabbits infused with high activities of the purified enzyme preparation. The plasma clearance of cathepsin D was also found to be both uniphasic and exponential (figures 1 and 7).

The infusion of high activities of exogenous cathepsin D into rabbits subjected to one hour of either mild (MABP = 40 mm Hg) or severe (MABP = 25 mm Hg) hemorrhagic

shock failed to modify physiologic responses in these shock models. Furthermore, all rabbits which received the purified cathepsin D preparation demonstrated highly significant elevations in plasma cathepsin D activity as compared to appropriate control rabbits. Previous studies have shown refractory hypotension to be a primary result of lysosomal extract infusions into experimental animals. This effect has been attributed both to a direct vasculotoxic action and to a direct cardiodepressant action resulting in a decreased cardiac output. Subsequent increases in total peripheral resistance, with enhanced splanchnic vasoconstriction, resulted in the compromise of the gastrointestinal tract and progression of the shock syndrome. In the present investigation, MABP, heart rate and temperature did not vary significantly between rabbits which received cathepsin D and comparable controls in either shock model. Of particular significance is the fact that rabbits infused with purified cathepsin D were, on the average, able to respond appropriately to the reinfusion of shed blood. In the mild shock group, rabbits which received cathepsin D developed a MABP of 85.2 $\pm$ 3.7 mm Hg 15 minutes after the reinfusion of blood and maintained a MABP to 71.0 $\pm$ 2.9 mm Hg at one hour following blood reinfusion. This response was strikingly similar to control rabbits which developed a MABP of 85.1 $\pm$ 8.6 mm Hg at 15 minutes and 74.7 $\pm$ 4.6 mm Hg at one hour after blood reinfusion. In the severe shock group, rabbits infused with cathepsin D demonstrated a MABP of 80.0 $\pm$ 5.6 mm Hg 15 minutes after blood reinfusion and a

MABP of 71.4 ± 7.1 mm Hg at one hour following the reinfusion of shed blood. Similarly, controls demonstrated a MABP of 81.0 ± 4.5 mm Hg at 15 minutes and 63.5 ± 4.7 mm Hg at one hour after blood reinfusion. Moreover, no statistically significant difference in mortality or mean survival time was demonstrated in either shock model between rabbits which received cathepsin D and control rabbits.

The lack of direct cardiodepressant actions of cathepsin D was confirmed by observations of the effect of this enzyme on isolated isometrically contracting rabbit myocardial muscle. Increasing concentrations of cathepsin D were tested for inotropic activity up to a maximum concentration of 40 units/cc. This concentration (equivalent to 114 units/mg protein specific activity) represented approximately three times the plasma cathepsin D activity usually observed in rabbits following one hour of severe hemorrhagic shock. Inotropic effects of cathepsin D were compared to those of the prepared control solution and the Krebs-Henseleit standard buffer solution. At the more dilute concentrations, the addition of purified cathepsin D extracts to isolated rabbit myocardial muscle failed to alter significantly the ability of these muscles to develop appropriate tensions following electrical stimulation. Cathepsin D at maximal concentrations produced significant positive inotropic effects for the first three minutes following its application to the myocardial muscle preparation. Thereafter, positive inotropy diminished. Furthermore, the inotropic effects of cathepsin D at the

various concentrations did not vary significantly from those of the corresponding prepared control solutions which followed similar patterns.

The appearance of cathepsin D in the circulation of animals subjected to one hour of either mild or severe hypotension was found to be highly predictable and reflected both the duration and severity of shock. In all rabbits studied, plasma cathepsin D demonstrated progressive increases in activity during the course of hypotension. In severely shocked rabbits, the rate of appearance was greater than that in animals subjected to an equal period of mild hypotension, and significantly higher levels of plasma cathepsin D activity were observed (figure 8). However, no significant correlation could be made between plasma cathepsin D levels and mortality. Neither rabbits which developed high endogenous levels of cathepsin D during shock, nor those infused with high activities of cathepsin D following shock, demonstrated correlations in these parameters.

Increased circulating activities of lysosomal hydrolases have been shown to be an early consequence of ischemia. Riciutti[61] found a significant loss of acid phosphatase from canine papillary muscle as early as one hour following coronary artery occlusion in the dog. Spath et al[62] demonstrated the loss of cathepsin D from the myocardium of cats within five hours after coronary artery occlusion. Following one hour of various degrees of hemorrhagic shock in the rabbit, Courtice et al[36] found significant increases in the plasma

activities of cathepsin D, ribonuclease and beta-glucuronidase. In the present study, elevations in plasma cathepsin D levels were found to occur very early in the course of hemorrhagic shock. All animals in every group demonstrated small increases in activity within 15 minutes after the induction of shock. Following 30 minutes of hemorrhagic shock, plasma cathepsin D activities were significantly elevated over preshock values. Twelve rabbits subjected to mild shock (MABP = 40 mm Hg) demonstrated an increase in plasma cathepsin D from a control value of 1.83 $\pm$ 0.49 units/mg protein to 5.91 $\pm$ 1.03 units/mg protein after 30 minutes of hypotension ($p < 0.01$). Over the same interval, five severely shocked rabbits (MABP = 25 mm Hg) exhibited an increase from an initial 1.65 $\pm$ 0.35 units/mg protein to 16.73 $\pm$ 2.72 units/mg protein ($p < 0.001$). This very early appearance of cathepsin D in shock plasma lends considerable support to the hypothesis that the solubilization of lysosomal membranes occurs early in the course of shock. Since cathepsin D is known to have a distribution limited to lysosomes, it is not unreasonable to assume that the early appearance of cathepsin D in the circulation during hemorrhagic shock must reflect equally early changes in the integrity of lysosomal membranes.

Some investigators have attributed a direct vasculotoxic action to lysosomal cathepsins, but adequate evidence for this effect is lacking. Following the discovery of Cohn and Hirsch[63] that the cytoplasmic granules of rabbit peritoneal exudate polymorphonuclear leukocytes are lysosomal in nature,

direct tests of the capacity of these granules to produce vasculotoxic and inflammatory effects have been undertaken. Thomas[21] succeeded in producing Swartzman-like lesions in rabbit skin using these rabbit peritoneal exudate leukocyte lysosomes and suggested that this effect resulted from the action of lysosomal cathepsins. The intradermal injection of these preparations into rabbit skin was followed by the intravenous administration of E-coli endotoxin. This resulted in extensive cutaneous lesions of hemorrhagic necrosis which were indistinguishable from the usual Swartzman reaction produced by preparing skin with endotoxin. Thomas' observations were confirmed by Halpern[64] who further demonstrated that protease inhibitors block the Swartzman-like lesions produced by polymorphonuclear leukocyte lysosomes, thus suggesting the importance of lysosomal proteases in the reaction. Burke et al[43] described a vascular permeability factor which was released from lysed polymorphonuclear lysosomes. The action of this factor was found to be completely blocked by the protease inhibitor trasylol.

Further studies have confirmed the vasculotoxic-inflammatory effects of lysosomal constituents but have failed to implicate the cathepsins as primary agents. Janoff et al[22] observed the actions of rabbit polymorphonuclear lysosomes, lysed by freezing-thawing, on rat and rabbit mesentery. The topical application of this preparation produced capillary permeability with petechial hemorrhages, stasis of blood flow and immigration of leukocytes in the mesenteric microcirculation.

These lysosomal materials were also studied following their separation into three fractions by precipitation in 20, 45, and 80 per cent ethanol. The 20 per cent ethanol precipitated fraction was found to produce all of the aforementioned injury reactions whereas the other fractions were inactive. Of significance is the fact that the active 20 per cent fraction contained no catheptic activity, which was found to be isolated in the 45 and 80 per cent fractions.

In the present study, the topical application of purified cathepsin D on rabbit mesentery failed to produce any significant vasculotoxic or inflammatory effects, thus confirming the observations of Janoff. However, a small but significant increase in vascular permeability, following the intradermal injection of purified cathepsin D into rabbit skin, was demonstrated using the Evans blue dye technique. Cathepsin D produced greater diameters of tissue bluing than either bacteriostatic saline or control solution, but also demonstrated a significantly smaller effect than histamine phosphate. These observations are of particular interest when compared to those of Golub and Spitznagel.[23] Using the Evans blue dye method, these investigators measured the ability of lysed polymorphonuclear lysosomes and 20, 45, and 80 per cent ethanol precipitated lysosomal fractions to produce increases in vascular permeability. Catheptic activity was found to be located primarily in the 45 per cent fraction, with lesser activities in the 80 per cent and no activity in the 20 per cent ethanol precipitated fractions. The intact lysosomes and 20 per cent

fractions gave large tissue bluing diameters in rabbit skin of 14 mm and 11 mm respectively, which compared with the 10.6 ± 1.6 mm diameters caused by histamine in the present study. The 45 and 80 per cent ethanol precipitated fractions demonstrated respective diameters of 3 mm and 2 mm, which were similar to the 3.0 ± 0.7 mm diameters produced by purified cathepsin D. It would therefore appear that cathepsin D has very little direct vasculotoxic activity as compared to other lysosomal constituents.

The present study adequately demonstrates that the administration of high activities of exogenous cathepsin D in experimental animals does not cause the cardiovascular disturbances seen in shock states or modify the course of experimental hemorrhagic shock. The enzyme or products of its action do not have direct vasculotoxic or vasoactive effects which were apparent in the various experimental models employed in these studies. The liberation of cathepsin D into shock plasma therefore appears to be a phenomenon which does not constitute a critical event in the progression of hemorrhagic shock.

Probably the most likely explanation for the failure of cathepsin D to effect significant alterations following its introduction into the circulation is its dependence on pH. Cathepsin D has a distinctly acid pH optimum and at normal blood pH the enzyme is relatively inactive. Even during severe hemorrhagic shock, blood pH rarely falls into the range of cathepsin D activity. For example, in the severe hemorrhagic

shock model employed in the present study, arterial pH decreased to an average 7.2. At this pH level, cathepsin D would at best express only a few per cent of its maximum activity. However, as a result of its efficient buffer system, blood pH does not accurately reflect changes in pH at the cellular level during hemorrhagic shock. Lemieux et al[65] found that the surface pH of skeletal muscle averaged 6.84 as compared to a mean blood pH of 7.35 in dogs subjected to six hours of graded hemorrhage to a shed blood volume of 50 per cent. Moreover, Silver, using intracellular micro pH electrodes, has shown that the liver cell pH declines from its normal mean value of 7.1 to 5.9 after six hours of severe (MABP = 30 mm Hg) hemorrhagic shock in the dog. These intercellular and intracellular pH levels approach the pH optimum of cathepsin D (as well as most other hydrolytic lysosomal enzymes), which is approximately 3.5 to 5.0. Therefore, following its release from lysosomes, cathepsin D in these environments might play a role in injury reactions and thus contribute to the propagation of pathophysiologic alterations in hemorrhagic shock.

There is presently no direct experimental evidence which demonstrates injurious actions of cathepsin D at the cellular level during shock. However, lysosomal constituents in general have been found capable of such effects. Concentrated lysosomal enzyme preparations have been shown to hydrolyse mitochondrial and microsomal membranes in vitro, causing increased membrane permeability.[67,68,69] One enzyme,

phospholipase C, has been found to have this action by hydrolysis of membrane phospholipids with the release of fatty acids. Mellors[70] has postulated that these released fatty acids cause further membrane destabilization by changing the surface-active properties of organelle membranes. Lysed whole lysosomal extracts have also been shown to inhibit mitochondrial energy-linked reactions. Mela et al[66] demonstrated in vitro inhibition of both ATPase activity and energy-linked ion transport as well as decreased respiratory control ratios in normal mitochondrial suspensions following the addition of lysosomal enzyme extracts. Moreover, at pH 6.5 these extracts significantly inhibited mitochondrial respirations as reflected by a nearly complete inhibition of stage 3 respiratory activity (measured as mitochondrial O_2 consumption in the presence of excess ADP and inorganic phosphate). These changes were further noted to reproduce precisely the mitochondrial defects induced in the liver by in vivo endotoxemic shock.

Cathepsin D is a potent acid protease capable of significant destructive effects in the intracellular environment that may be produced in circulatory shock. Following its release from lysosomes, and in adequate concentrations, cathepsin D could contribute to injurious reactions at the cellular level through digestion of intracellular membranes and enzymes and by modifications in cell walls and peripheries. Further investigations are necessary to elucidate the relationship of cathepsin D to cellular events occurring in the course of hemorrhagic shock.

V CONCLUSIONS

1. The method of Barrett for the isolation of cathepsin D from rabbit liver is reproducible and gives quantities of purified enzyme which are sufficient for physiologic study.

2. The plasma clearance of cathepsin D, following its infusion into normal rabbits, is both uniphasic and exponential with a half-life of approximately 90 minutes.

3. The appearance of cathepsin D in the circulation of experimental animals subjected to hemorrhagic shock is predictable and reflects both the duration and severity of shock.

4. Significant increases in plasma cathepsin D activity occur early in the course of experimental hemorrhagic shock. This suggests that disruption of normal lysosomal membrane function is an early event in the progression of circulatory shock.

5. The administration of high activities of exogenous cathepsin D in experimental animals does not cause the cardiovascular disturbances seen in circulatory shock or modify the course of experimental hemorrhagic shock. Furthermore, cathepsin D does not have direct cardiodepressant actions as assessed by the isolated rabbit myocardial assay and does not exhibit significant vasculotoxic or inflammatory effects. These findings suggest that cathepsin D does not cause adverse reactions following its introduction into the systemic circulation during hemorrhagic shock.

6. Cathepsin D is probably rendered inactive at the pH of blood normally seen in shock states.

7. It is postulated that cathepsin D may be damaging to intercellular and intracellular components as a result of the lowered pH found in these environments during circulatory shock.

VI FIGURES

LIST OF FIGURES

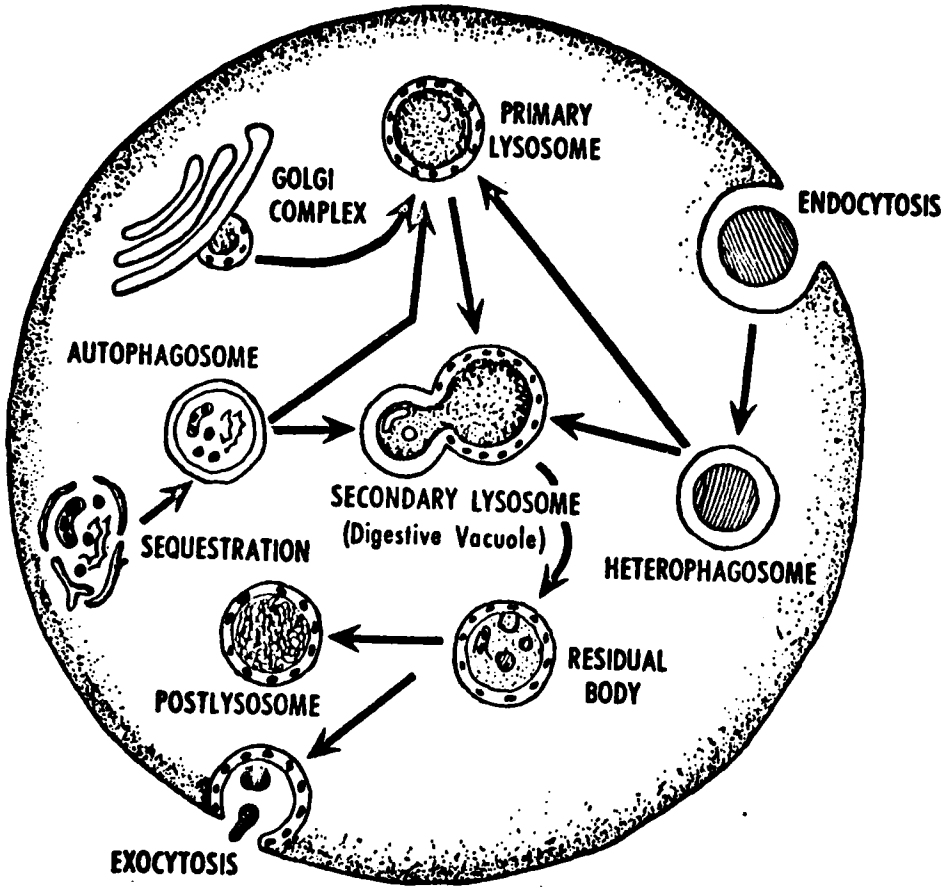

FIGURE 1: Schematic representation of normal lysosomal functions (modified from Goldstein[30] and Lazarus[7]).

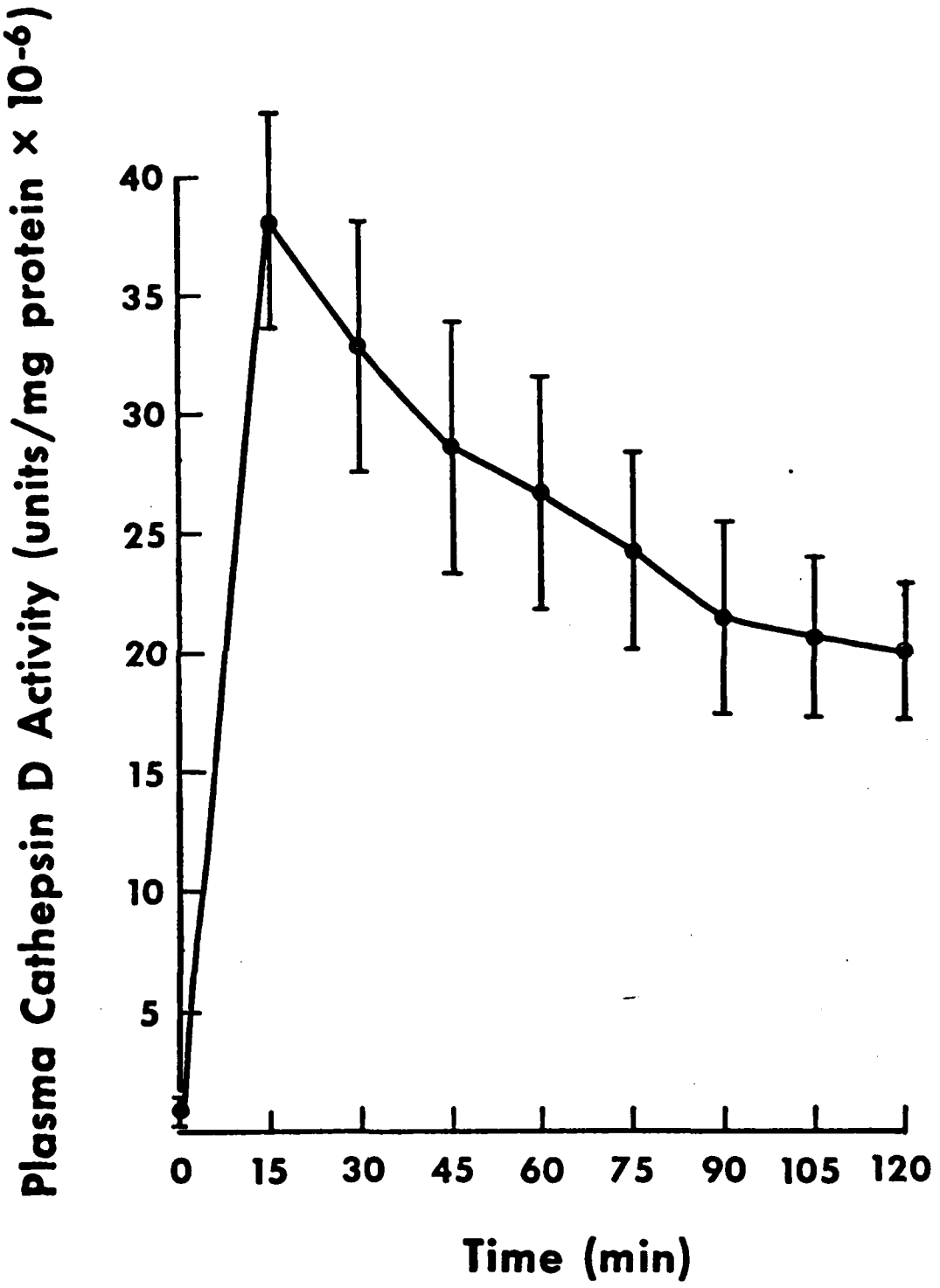

FIGURE 2: Plasma cathepsin D activities vs. time after a single infusion of a purified cathepsin D extract into normal rabbits. Each point represents the mean value of seven rabbits. Brackets indicate standard errors of the mean (SEM).

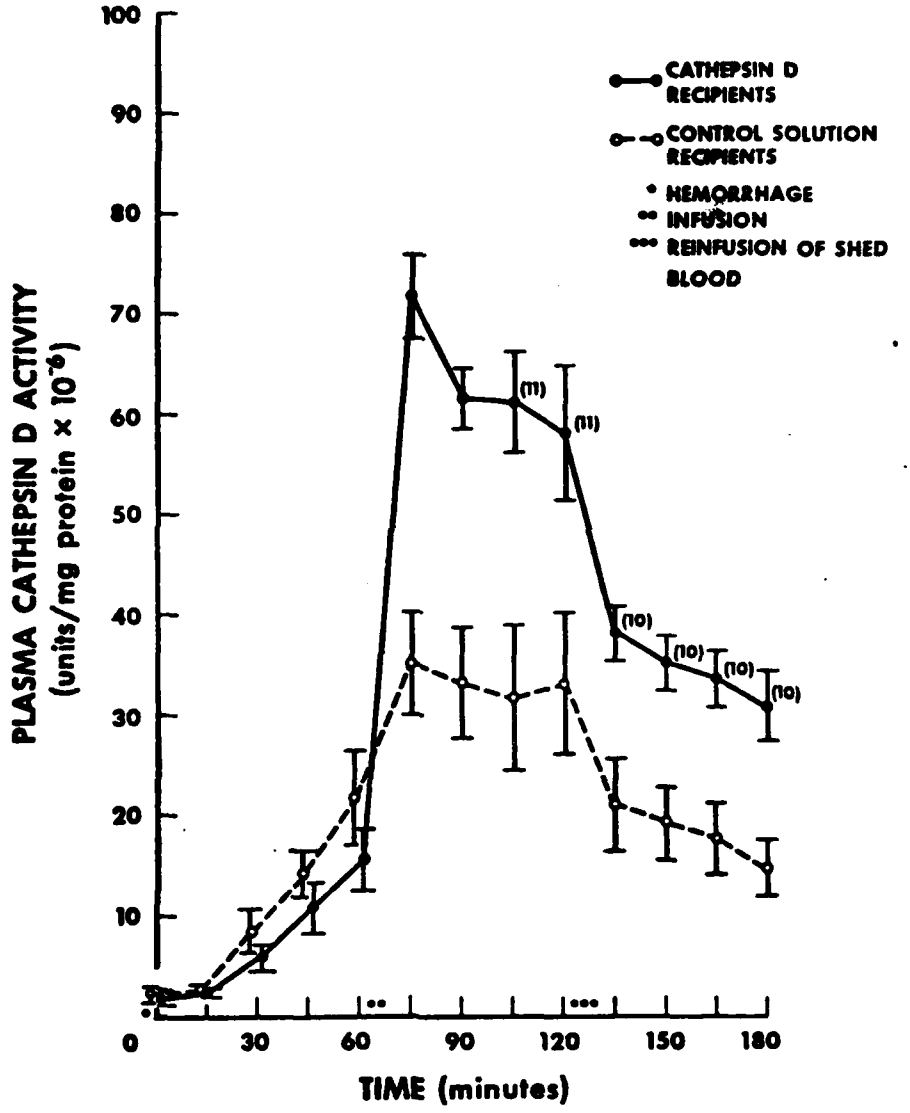

FIGURE 3: Plasma cathepsin D activities vs. time in rabbits subjected to mild (MABP = 40 mm Hg) hemorrhagic shock, infusion of a purified cathepsin D extract or control solution and reinfusion of shed blood. Each point represents the mean value of 12 rabbits except where otherwise indicated in parentheses. Brackets indicate standard errors of the mean (SEM).

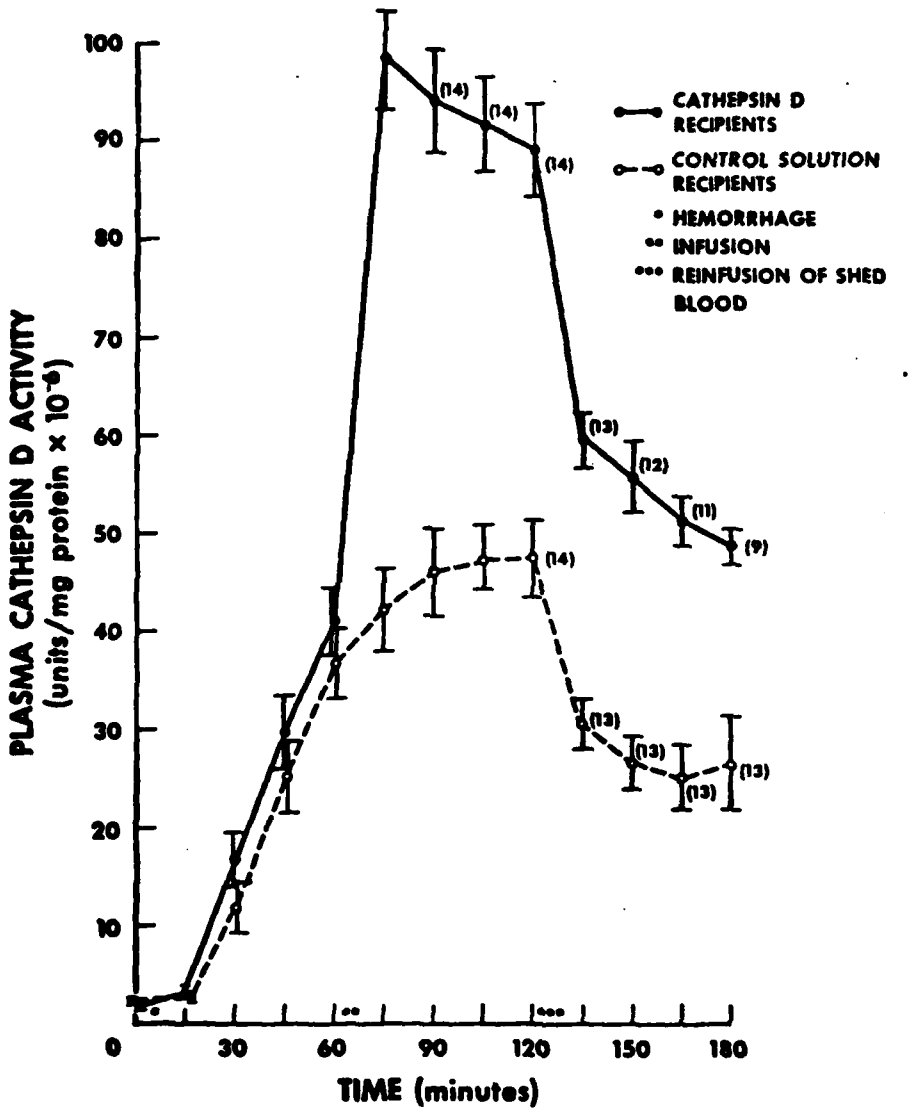

FIGURE 4: Plasma cathepsin D activities vs. time in rabbits subjected to severe (MABP = 25 mm Hg) hemorrhagic shock, infusion of a puriried cathepsin D extract or control solution and reinfusion of shed blood. Each point represents the mean value of 15 rabbits except where otherwise indicated in parentheses. Brackets indicate standard errors of the mean (SEM).

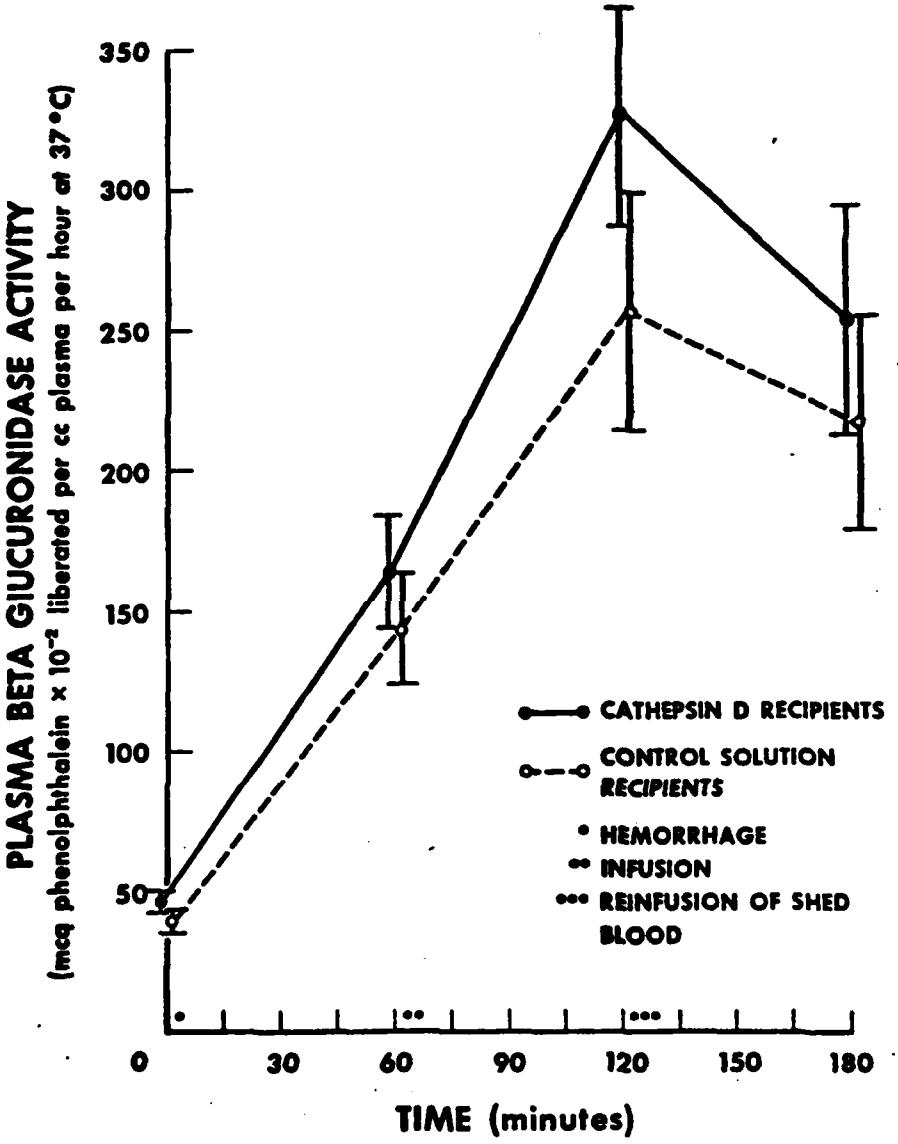

FIGURE 5: Plasma beta-glucuronidase activities in rabbits subjected to severe (MABP = 25 mm Hg) hemorrhagic shock, infusion of a purified cathepsin D extract or control solution and reinfusion of shed blood. Each point represents the mean value of nine rabbits. Brackets indicate standard errors of the mean (SEM).

FIGURE 6: Physical appearance of cathepsin D extract at successive stages of purification: (A) Homogenate; (B) Homogenate after addition of calcium chloride dihydrate; (C) Incubated supernatant; (D) Supernatant following acetone fractionation and centrifugation; (E) Redispersed precipitate after second acetone fractionation and filtration; (F) Dialized and filtered extract; (G) DEAE cellulose column extract; (H) Final preparation after CM column chromatography. Infusates used in previous studies had a physical appearance between stages B and C.

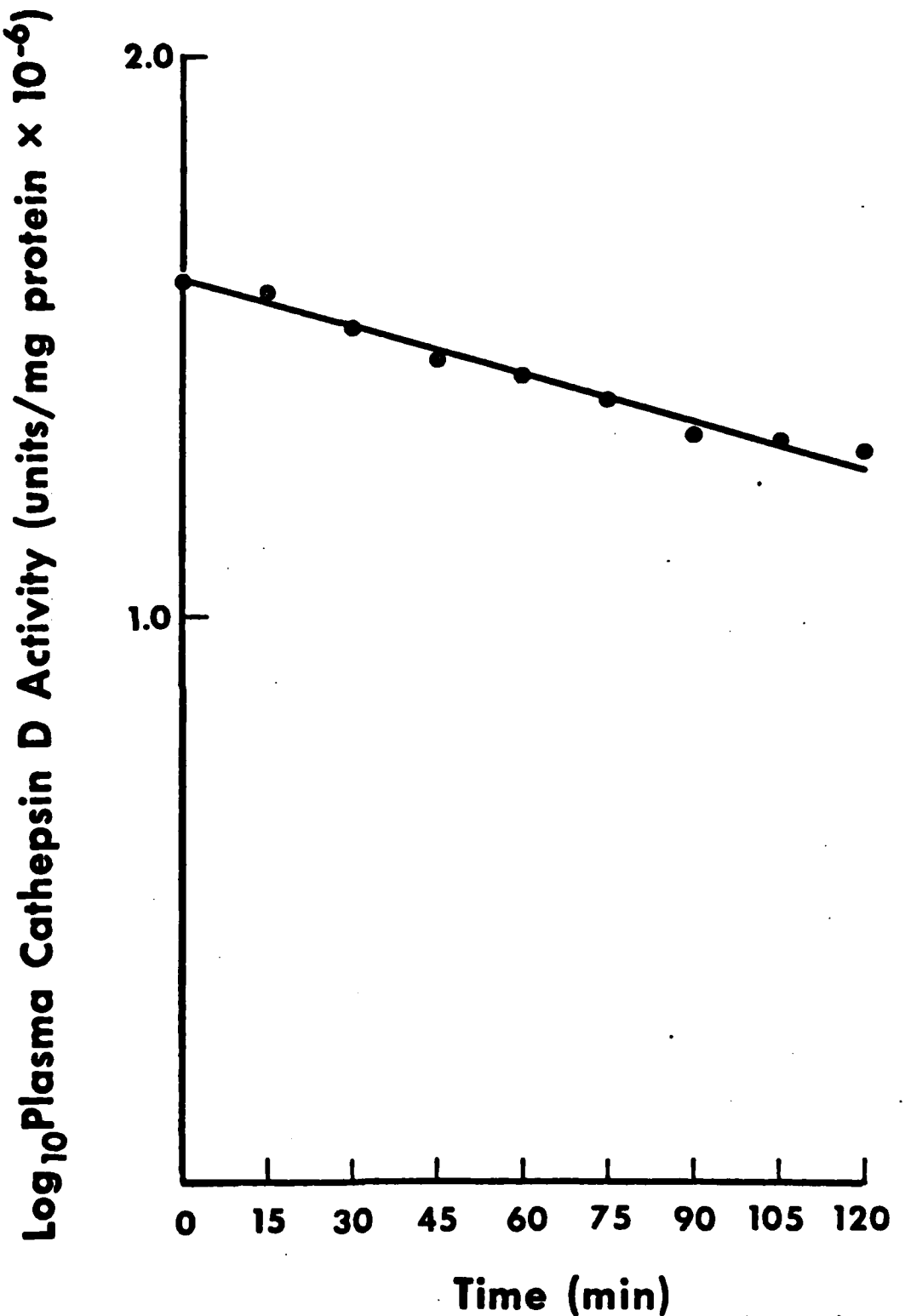

FIGURE 7: Semilogarithmic plot of plasma cathepsin D disappearance following a single infusion of a purified enzyme extract into seven normal rabbits. Plot is along a least squares line of best fit (correlation coefficient = 0.99).

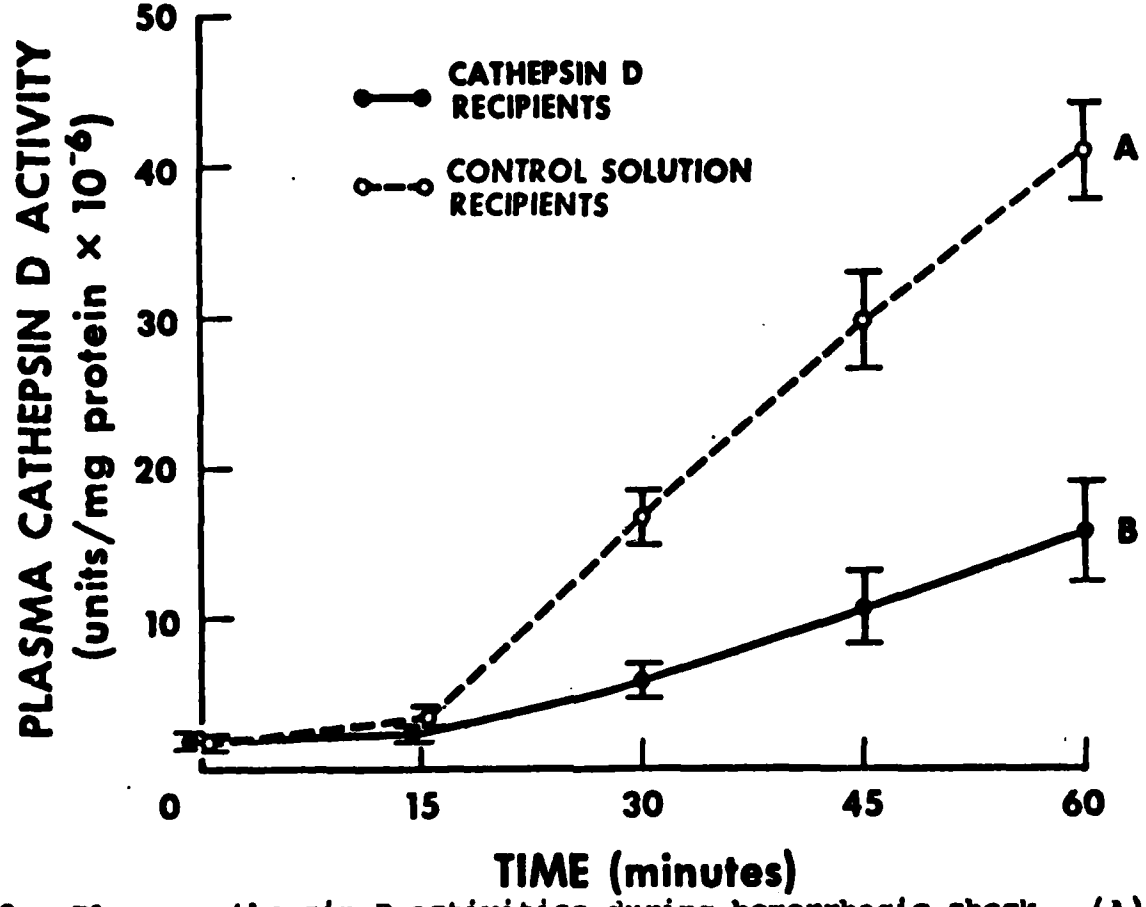

FIGURE 8: Plasma cathepsin D activities during hemorrhagic shock. (A) Enzyme activities of 15 rabbits subjected to severe (MABP = 25 mm Hg) shock for one hour prior to the infusion of a control solution. (B) Enzyme activities of 12 rabbits subjected to mild (MABP = 40 mm Hg) shock for one hour prior to the infusion of a purified cathepsin D extract.

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TABLE 1

PROCEDURAL ANALYSIS OF CATHEPSIN D PURIFICATION

	Relative Activity (units/ml)	Specific Activity (units/mg protein)	Yield (%)	Purification (fold)
Homogenate	101.1 ± 13.9	0.6 ± 0.3	100	1
Dialized Extract	59.3 ± 5.2	39.3 ± 3.8	59	66
DEAE Cellulose Column Extract	51.4 ± 4.6	77.2 ± 11.5	51	129
CM Cellulose Column Extract	44.9 ± 4.9	121.8 ± 22.9	44	203

All values are means ± SEM. Results are based on eight purification procedures. Percentage yield and purification are estimated from relative and specific activities respectively.

TABLE 2

BIOCHEMICAL ANALYSIS OF FINAL PURIFIED CATHEPSIN D AND CONTROL SOLUTIONS

	Cathepsin D Preparation	Control Solution
Cathepsin D	121.8 ± 22.9 units/mg	0 ± 0
beta-glucuronidase	0 ± 0	0 ± 0
Acid Phosphatase	0 ± 0	0 ± 0
Alkaline Phosphatase	5.5 ± 3.3 miu/ml	1.0 ± 0.6
Lactic Dehydrogenase	6.2 ± 6.2 miu/ml	3.3 ± 0.9
Glutamic Oxaloacetic Acid Transaminase	4.2 ± 2.1 miu/ml	7.0 ± 2.0
Gamma Glutamyl Transpeptidase	0.7 ± 0.3 miu/ml	0.0 ± 0.0
Sodium	192.1 ± 8.3 meg/L	195.7 ± 4.2
Potassium	0 ± 0	0 ± 0
Chloride	0 ± 0	0 ± 0
Calcium	0.6 ± 0.3 mg%	0.4 ± 0.1
Glucose	5.5 ± 2.2 mg%	2.0 ± 1.0
Uric Acid	0.3 ± 0.2 mg%	0.7 ± 0.3
Cholesterol	13.0 ± 1.4 mg%	7.3 ± 3.2
Triglycerides	0 ± 0	0 ± 0
Total Bilirubin	0.02 ± 0.02 mg%	0.03 ± 0.03

Results are based on eight purification procedures.
All values are means ± SEM.

TABLE 3

PHYSIOLOGIC RESPONSES OF NORMAL RABBITS
INFUSED WITH PURIFIED CATHEPSIN D

Interval (min.)	MABP (mmHg)	Heart Rate (beats/min.)	Temperature (°C)
0+	88.4 ± 2.5	239 ± 14	38.1 ± 0.1
15‡	91.7 ± 3.0	222 ± 14	37.9 ± 0.1
30	87.7 ± 3.4	218 ± 10	38.1 ± 0.3
45	85.1 ± 3.9	215 ± 12	37.8 ± 0.1
60	87.9 ± 3.9	221 ± 15	37.1 ± 0.1*
75	88.0 ± 4.4	224 ± 12	37.6 ± 0.1*
90	86.9 ± 3.4	240 ± 10	37.6 ± 0.1*
105	86.3 ± 2.5	240 ± 8	37.6 ± 0.2*
120	86.4 ± 3.5	251 ± 9	37.6 ± 0.2*

All values are means ± SEM

+ Prior to infusion of cathepsin D

‡ Following infusion of cathepsin D

* p .005

TABLE 4

PHYSIOLOGIC RESPONSES OF RABBITS SUBJECTED TO MILD HEMORRHAGIC SHOCK PRIOR TO A SINGLE INFUSION OF A PURIFIED CATHEPSIN D EXTRACT

INTERVAL (min)	N	MABP (mmHg)	HEART BEAT (beats/min)	TEMPERATURE (°C)	pH
0 +	12	84.4 ± 3.8	212 ± 8	37.1 ± 0.3	7.47 ± 0.03
	12	88.9 ± 1.8	243 ± 16	37.9 ± 0.2	7.44 ± 0.02
15 ++	12	40.6 ± 0.8	201 ± 9	37.8 ± 0.3	
	12	38.6 ± 0.8	218 ± 15	38.0 ± 0.2	
30	12	40.0 ± 0.5	206 ± 11	37.7 ± 0.3	
	12	38.6 ± 0.8	224 ± 10	38.0 ± 0.2	
45	12	39.7 ± 0.7	229 ± 12	37.7 ± 0.4	
	12	38.9 ± 0.7	243 ± 9	38.0 ± 0.2	
60	12	39.9 ± 0.4	245 ± 9	37.6 ± 0.4	7.36 ± 0.03
	12	39.0 ± 0.5	273 ± 5	37.8 ± 0.2	7.32 ± 0.04
75 *	12	44.1 ± 1.6	255 ± 10	37.4 ± 0.5	
	12	43.1 ± 3.0	278 ± 9	37.6 ± 0.3	
90	12	42.7 ± 1.2	255 ± 10	37.3 ± 0.5	
	12	43.6 ± 2.9	283 ± 8	37.6 ± 0.3	
105	11	38.0 ± 1.9	254 ± 9	37.2 ± 0.5	
	12	40.1 ± 2.7	273 ± 12	37.6 ± 0.4	
120	11	35.7 ± 1.3	253 ± 11	37.5 ± 0.6	7.42 ± 0.02
	12	37.3 ± 2.1	275 ± 14	37.6 ± 0.4	7.37 ± 0.07
135 **	11	85.2 ± 3.7	228 ± 16	37.8 ± 0.7	
	12	85.1 ± 8.6	254 ± 14	37.3 ± 0.4	
150	10	79.3 ± 3.3	229 ± 14	36.7 ± 0.6	
	12	84.0 ± 6.5	258 ± 12	37.3 ± 0.4	
165	10	74.2 ± 2.3	234 ± 14	36.7 ± 0.6	
	12	79.6 ± 5.3	270 ± 13	37.4 ± 0.5	
180	10	71.0 ± 2.9	237 ± 11	36.9 ± 0.6	7.45 ± 0.02
	12	74.7 ± 4.6	267 ± 10	37.3 ± 0.5	7.43 ± 0.06

Note: Results in rabbits infused with Cathepsin D are listed above results in control rabbits. All values are means ± SEM.

\+ Prior to Bleeding.
++ Hemorrhagic Shock.
* $\bar{P}$ infusion of Cathepsin D or Control Sclution.
** $\bar{P}$ reinfusion of Shed Blood.

TABLE 5

PHYSIOLOGIC RESPONSES OF RABBITS SUBJECTED TO SEVERE HEMORRHAGIC SHOCK PRIOR TO A SINGLE INFUSION OF A PURIFIED CATHEPSIN D EXTRACT

INTERVAL (min)	N	MABP (mmHg)	HEART BEAT (beats/min)	TEMPERATURE (°C)	pH
0 +	15	84.1 ± 1.9	246 ± 7	38.2 ± 0.1	7.48 ± 0.01
	15	85.3 ± 3.2	250 ± 8	38.2 ± 0.1	7.50 ± 0.01
15 ++	15	25.9 ± 0.4	207 ± 7	38.3 ± 0.1	
	15	25.1 ± 0.2	219 ± 7	38.2 ± 0.1	
30	15	25.7 ± 0.3	226 ± 9	38.2 ± 0.1	
	15	25.5 ± 0.2	248 ± 8	38.1 ± 0.1	
45	15	25.5 ± 0.5	252 ± 7	38.1 ± 0.1	
	15	25.1 ± 0.1	257 ± 7	38.0 ± 0.1	
60	15	25.4 ± 0.3	254 ± 7	37.9 ± 0.1	7.20 ± 0.03
	15	25.1 ± 0.2	258 ± 8	37.8 ± 0.1	7.23 ± 0.03
75 *	15	38.1 ± 1.2	263 ± 8	37.6 ± 0.2	
	15	35.3 ± 2.3	262 ± 7	37.4 ± 0.1	
90	14	37.1 ± 1.4	269 ± 9	37.5 ± 0.2	
	15	32.2 ± 2.4	261 ± 8	37.4 ± 0.1	
105	14	32.4 ± 1.9	268 ± 9	37.5 ± 0.2	
	15	30.6 ± 2.4	250 ± 8	37.3 ± 0.1	
120	14	27.9 ± 1.6	254 ± 10	37.5 ± 0.2	7.21 ± 0.03
	14	28.9 ± 2.3	249 ± 9	37.2 ± 0.1	7.23 ± 0.05
135 **	13	80.0 ± 5.6	257 ± 8	36.9 ± 0.2	
	13	81.0 ± 4.5	238 ± 7	36.6 ± 0.2	
150	12	72.0 ± 7.8	248 ± 8	36.9 ± 0.2	
	13	72.3 ± 4.9	241 ± 7	36.7 ± 0.2	
165	11	66.1 ± 7.0	244 ± 6	37.0 ± 0.2	
	13	59.5 ± 4.7	244 ± 7	36.8 ± 0.2	
180	9	71.4 ± 7.1	255 ± 6	37.0 ± 0.2	7.41 ± 0.05
	13	63.5 ± 4.7	243 ± 8	36.8 ± 0.2	7.38 ± 0.04

Note: Results in rabbits infused with Cathepsin D are listed above results in control rabbits. All values are means ± SEM.

+ Prior to Bleeding. * $\bar{P}$ infusion of Cathepsin D or Control Solution.

++ Hemorrhagic Shock. ** $\bar{P}$ reinfusion of Shed Blood.

TABLE 6

EFFECTS OF PURIFIED CATHEPSIN D ON VASCULAR PERMEABILITY OF RABBIT SKIN

	Diameter of Blue Tissue (mm)	Intensity of Bluing (0 to ++++)	Significance Level
Purified Cathepsin D	3.0 ± 0.7	++	----
Control Solution	0.9 ± 0.4	++	*p .02
Bacteriostatic Saline	0.3 ± 0.2	++	p .001
Histamine Phosphate	10.6 ± 1.6	++++	p .001

All values are means ± SEM

* Probability values are comparisons to the mean diameter of tissue bluing of cathepsin D.

TABLE 7

INOTROPIC ACTIVITY OF PURIFIED CATHEPSIN D
IN ISOLATED RABBIT MYOCARDIAL MUSCLES

*Concentration	Interval (min)	**Per Cent Change in Developed Tension After Cathepsin D	**Per Cent Change in Developed Tension After Control Solution
Maximal (Undiluted)	1	+130.6 ± 36.0	+121.2 ± 34.5
	2	+ 81.6 ± 34.1	+ 58.4 ± 38.4
	3	+ 62.0 ± 37.1	+ 49.0 ± 37.2
	4	- 8.3 ± 19.5	+ 9.3 ± 27.3
1:5	1	+ 47.0 ± 19.1	+ 46.0 ± 14.0
	2	+ 14.3 ± 9.8	+ 19.0 ± 11.6
	3	- 10.0 ± 10.0	- 0.7 ± 11.0
	4	- 13.5 ± 6.5	- 3.5 ± 3.5
1:10	1	+ 10.3 ± 1.9	+ 16.3 ± 10.5
	2	+ 4.7 ± 4.7	+ 8.3 ± 9.2
	3	- 11.7 ± 8.4	- 1.0 ± 5.5
	4	- 12.7 ± 7.8	- 12.0 ± 2.0

*Cathepsin D Concentrations: Maximal, 40 units/cc; 1:5, 8 units/cc; 1:10, 4 units/cc

**All values are means ± SEM

***Per cent change represents the difference in developed muscle tension between the sample and a Krebs-Henseleit buffer standard.

Differences in per cent change between cathepsin D and control solution are insignificant at all corresponding intervals.

VIII BIBLIOGRAPHY

BIBLIOGRAPHY

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